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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

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TITLE OF THE INVENTION *(280 characters max)*

PSEUDOTYPED RETROVIRAL VECTOR FOR GENE THERAPY OF CANCER

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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	A check or money order is enclosed to cover the Provisional filing fees		PROVISIONAL FILING FEE AMOUNT (S)	$150.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

SIGNATURE [signature]

Date 04/23/1999

TYPED or PRINTED NAME France Côté

REGISTRATION NO. 37,037

[ ] Additional inventors are being named on separately numbered sheets attached hereto

PROVISIONAL APPLICATION FILING ONLY

PSEUDOTYPED RETROVIRAL VECTOR FOR GENE THERAPY OF CANCER

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to retroviral expression vectors and more particularly to pseudotyped retroviral vectors for gene therapy of cancer.

(b) Description of Prior Art

Tumor cells modified to express the Herpes Simplex Virus Thymidine Kinase gene (TK) acquire the ability to convert the non-toxic nucleobase analog gancyclovir (GCV) to its cytotoxic metabolite gancyclovir-phosphate. Cells genetically engineered to express this "suicide" gene are eliminated if exposed to gancyclovir. Experimental brain tumor implants consisting of a mixture of unmodified tumor cells with TK-expressing cells also regress following gancyclovir treatment without harm to adjacent normal tissue. This phenomena, where a minority of TK-expressing cells lead to the death and elimination of adjacent tumor cells not expressing TK, has been termed the "bystander effect".

The "bystander" effect is dependent, in part, on cell-cell contact and on intercellular communications - gap junctions - through which gancyclovir-phosphate can circulate between TK-positive and TK-negative tumor cells. Phagocytosis of gancyclovir-phosphate laden cell debris by adjacent tumor cells also leads to cell death. Blood vessel endothelial cells within or adjacent to the tumor may also acquire TK, and their destruction with gancyclovir therapy, thus, may also contribute to tumor regression. "Suicide" tumors release inflammatory cytokines which promote hemorrhagic necrosis in local, but non-contiguous, tumor deposits. Furthermore, tumors undergoing a necrotic death, as opposed to apoptosis, will up-regulate the expression of proteins such as hsp70, IL10 and IL12, which may enhance immune recognition and rejection. Necrotic tumors may be infiltrated with a wide assortment of immunocompetent cells such as CD4+ lymphocytes, CD8+ lymphocytes, NK cells and Antigen Presenting Cells. These infiltrating cells may take part in a tumor-specific immune response which is an important component of the local (12) as well as distant anti-tumor immune

bystander effect. Intracerebral tumors are also susceptible to immune clearance following suicide gene expression, suggesting that the brain is not an immune sanctuary for cancer. Therefore, tumor-targeted suicide gene delivery leads to eradication of a defined tumor deposit if a sufficient number of targeted cells express the suicide gene. Malignant brain tumors are an appealing target for suicide gene delivery, since the entire malignancy is confined to the brain and amenable to eradication by the bystander effect. Key components for the success of this strategy are the genetic vector from which the suicide gene is expressed and its delivery vehicle.

Viral vectors remain the most efficient means to introduce genetic material in tumor cells *in vivo*. This is usually achieved by direct intra-tumoral or intravenous injection of a viral particle suspension. Among viral vector delivery platforms, adenoviruses are among the most studied for tumor-targeted gene delivery. Adenoviruses can be concentrated to high titers, which facilitates delivery of large viral doses to tumors However, because of their ability to disseminate beyond local injection site and to transduce contiguous normal brain, including astrocytes, neurons and ependymal cells, suicide gene expression may lead to significant toxicity following gancyclovir treatment.

Recombinant retroviral vectors are well characterized as vehicles for tumor-targeted gene delivery. Retroviruses can integrate only in cells undergoing mitosis shortly after infection (23). Quiescent cells - such as normal brain tissue adjacent to a targeted tumor deposit - will be refractory to gene transfer and spared from subsequent toxicity (4). For this reason, retroviral vectors have been extensively used in human clinical trials studying suicide gene delivery to malignant brain tumors. Limitations to the use of retroviruses are: their inability to infect cells which do not express the retroviral receptor and, the low particle concentration in clinical-grade viral preparations. Clinical-grade retroviral particle preparations usually have titers $<10^7$ particles/ml. Assuming that a target tumor having a 1 cm diameter contains at least 10^8 cells, it would be necessary to inject intra-tumorally at least >10 ml of viral particle preparation to deliver an equal number of viral particles. This logistical impediment to retroparticle

delivery has been addressed by directly injecting murine retroviral producer cells (VPCs) in to tumors *in vivo*, the idea being that locally produced viral particles could transduce cancer cells. Though this gene delivery approach led to cures in a rat model of brain cancer, this was probably achieved as a consequence of delivering as many VPCs as there were tumor cells (4). In human clinical trials, where this strategy was duplicated by injecting amphotropic VPCs with a titer of $1x10^{5}$ cfu/ml, low - albeit detectable - TK gene transfer efficiency was noted in tumor cells. Furthermore, a specific immune response against VPCs was elicited (30). Although "suicide" retrovectors are "safe", implantation of VPCs as a means to deliver retroparticles is of limited efficacy. Poor suicide gene transfer to tumor cells is a major impediment to therapeutic utility.

Retroparticles which incorporate the Vesicular Stomatitis Virus G (VSVG) protein differ from traditional murine retroviral pseudotypes by their high affinity for a wide assortment of eukaryotic cells. This is primarily due to the ability of VSVG to recognize membrane phospholipid as a minimal receptor. Unlike standard murine retroviruses, VSVG retrovectors are also relatively resistant to deactivation by human complement (34). Furthermore, like adenoviruses, VSVG-typed retroviruses can be concentrated to high titers by centrifugation and frozen/thawed without loss of activity. The VSVG pseudotype does not alter the retroviral genome's restricted targeting of cycling cells.

It would be highly desirable to be provided with a suitable delivery vehicle for suicide gene transfer, combining high titer, particle stability and tumor-specificity

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a suitable retroviral vector for gene therapy of a cancer.

For example, VSVG-typed retroparticles may be suitable for delivering a therapeutic gene to a tumor tissue.

For example, the cancer may be a brain cancer.

Examples of therapeutic genes include suicide genes

For example, an HSVTK-expressing retrovector and VSVG-pseudotyped retroparticles were constructed. Human glioma cell lines can be transduced *in vitro* and express functionally significant amounts of HSV TK. Concentrated retroparticles were administered intra-tumorally in a rat model of brain cancer and a significant survival benefit was noted following gancyclovir therapy.

The retrovector may incorporate the AP2 expression vector. The AP2 expression vector allows for a high level expression of a transgene and incorporates a reporter gene for monitoring of the transgene expression *in vitro* and *in vivo*. Furthermore, the reporter protein allows for a sorting of producer cells and facilitates the measurement of the retroviral titer.

In accordance with the present invention, retrovectors which may be used include, without limitation, AP2 expression vector, AP2 derivatives thereof such as its first derivative AP3 which includes the HSVRK suicide gene. Other derivatives include MD1 which is a AP2 derivative which incorporates human GMCSF. JGH2 which expresses a novel GFP-HSVTK fusion protein, JGH2 derivatives thereof which incorporate immunomodulatory genes as well as the GFP/TK fusion protein. AP2 derivatives incorporate genes of therapeutic interest for the treatment of cancer.

Preferred AP2 expression vector derivatives include, without limitation, the following:

HSV thymidine kinase (AP3);
GMCSF (MD1);
RARB2,
IRF3;
IRF3-5d;
MCP1;
Rantes,
MIP1alpha;
MIP1beta;
MCP1.

Preferred JGH2 expression vector derivatives include, without limitation, the following:

GMCSF (MD2);
IRF3 (AP6);

IRF3/5D (AP7).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Schematic representations of plasmids and retrovectors. Fig. 1A: AP2 plasmid retrovector serves as a template for the co-expression of the EGFP reporter and of a linked cDNA in eukaryotic cells. The cDNA of interest is inserted in the multiple cloning site upstream of the IRES. Fig. 1B: pTKiGFP is a derivative of AP2 which contains the HSVTK gene. Transfection of this plasmid into retroviral packaging cells will lead to the production of replication-defective retroparticles. Fig. 1C: Target cells transduced with vTKiGFP will integrate the retrovector in their genomic DNA. The DNA structure (flanked by LTRs) and coding sequences are depicted.

Fig. 2. Flow cytometric analysis of vTKiGFP transduced glioma cells. UWR7 human glioma cells were transduced with vTKiGFP and subsequently analyzed by flow cytometry for green fluorescence, as described in "Materials and Methods". GFP serves as a reporter of retrovector expression in transduced cells.

Fig. 3 Southern Blot analysis on vTKiGFP transduced glioma cells Following transduction with vTKiGFP, the retrovector will integrate into genomic DNA. Digest of genomic DNA with NheI, which cuts once in each flanking LTR, and subsequent probing of Southern blot with a vector complementary sequence will allow detection of integrated proviral sequences with a predicted size of 4kb (schematic at right). Left. Southern blot analysis of transduced (+) and untransduced (-) UWR7 cells with a GFP cDNA-specific probe, as described in "Materials and Method". Molecular weights are indicated.

Fig. 4: Growth suppression of human glioma cells with gancyclovir. The indicated human glioma cell lines were transduced with vTKiGFP (open squares) or the control retrovector vDHFRiGFP (open circle). These and untransduced controls (open diamonds) were subsequently exposed to gancyclovir for 6 days. and cell survival was measured by the MTT assay as described in "Materials and Methods". Percent survival is plotted against gancyclovir

concentration (log scale). Data points, mean survival measured in three separate experiments; bars, SD. SD smaller than data point icon are not displayed.

Fig. 5. Flow cytometric analysis of 293AP3 producer cells. 293GPG packaging cells were stably transfected with pTKiGFP and a Zeocin resistance plasmid. A mixed population of Zeocin resistant 293AP3 cells was generated and characterized for GFP expression by flow cytometry as described in "Materials and Methods". Percent GFP+ cells is indicated. These cells were subsequently utilized to generate vTKiGFP stock for concentration and *in vivo* delivery.

Fig. 6. Transduction of glioma cells with concentrated vTKiGFP retrovector stocks. vTKiGFP retroparticles were collected and concentrated to 84 and 1000 fold (volume/volume) as described in "Materials and Methods". 1X and 84X virus stock were diluted (as indicated on left) in a final volume of 1 ml and applied to $2.3x10^5$ UWR7 cells in a 24 well dish. Three days following a single application of vector, cells were analyzed for GFP expression by flow cytometry. Percent GFP+ is indicated in histogram figures. Dilutions of 1000X stock was applied to $5.4x10^5$ C6 glioma cells and analyzed three days later for GFP expression. Titer extrapolated from these experiments were: 1X: $2.9x10^7$ cfu/ml, 84X: 2.2x10^[illegible] cfu/ml. 1000X: $2.3x10^{10}$ cfu/ml.

Fig. 7 *In vivo* transduction of C6/lacZ tumors with vTKiGFP. Brain tumors were harvested post-mortem as described in Materials and Methods. TOP (panel A, B), tumor from a control rat which received vTKiGFP without subsequent treatment with GCV (rat was sacrificed on day 30 post tumor implantation due to morbid state). MIDDLE (Panel C, D), tumor from a control rat which did not receive vTKiGFP but was treated with GCV (rat was sacrificed on day 43) BOTTOM (Panel E, F), tumor from a test rat which received vTKiGFP and subsequent treatment with GCV which suffered symptomatic recurrent tumor (rat was sacrificed on day 32). GFP expression (panels A, C, E) was compared to subsequent histochemical staining of C6/lacZ tumor cells with the substrate X-gal (panels B, D, F). Magnification of 100X for all photomicrographs.

Fig. 8. Kaplan-Meier survival curve of rats with experimental glioma. Sprague-Dawley rats received 2×10^{4} C6/lacZ glioma cells by stereotactic injection in the right brain hemisphere as described in "Materials and Methods". Six days later, eighteen animals were administered 9μL of 1000x vTKiGFP stock in the same stereotactic coordinates as the previous C6/lacZ implant. 48 hours later, test animals (n=12) received GCV 50mg/kg twice daily for 5 days followed by 50mg/kg once daily for 5 more days. The other animals (n=6) were administered saline only. In a separate experiment, a supplementary cohort (n=5) received a C6/lacZ glioma implant followed 9 days later by GCV treatment (no retrovector administered). The survival seen in the test group (vTKiGFP + GCV) is significantly grater than that in either control groups ($p<0,001$ by Log rank). There is no significant difference in survival between the two control groups.

DETAILED DESCRIPTION OF THE INVENTION

Direct *in vivo* tumor-targeting with "suicide" viral vectors is limited by either inefficient gene transfer [i.e. retroviral vectors] or indiscriminate transfer of a conditionally toxic gene to surrounding non-malignant tissue [i.e. adenoviral vectors]. Retrovectors pseudotyped with the Vesicular Stomatitis Virus G protein (VSVG) may serve as a remedy to this conundrum. These retroviral particles differ from standard murine retroviruses by their very broad tropism and the capacity to be concentrated by ultracentrifugation without loss of activity. A VSVG-typed retrovector can be utilized for efficient and tumor specific Herpes Simplex Virus Thymidine Kinase (TK) gene delivery *in vivo*. A bicistronic retroviral vector which expresses TK and Green Fluorescence Protein (pTKiGFP) was constructed. The 293GPG packaging cell line was utilized to generate vTKiGFP retroparticles. In cytotoxicity assays, vTKiGFP-transduced human glioma cell lines were sensitized to the cytotoxic effects of gancyclovir (GCV) 10,000 fold. Subsequently, the virus was concentrated by ultracentrifugation to a titer of 2.3×10^{16} cfu/ml. The anti-tumor activity of vTKiGFP retroparticles was tested in a rat C6 glioma model of brain cancer. Concentrated retrovector stock (9μL volume) was injected stereotactically in pre-established intra-cerebral tumor. Subsequently, rats were treated with GCV for 10 days. Control rats (no GCV)

had a mean survival of 38 days (range 20-52 days). Sections performed on post-mortem brain tissue revealed large tumors with evidence of high efficiency retrovector transfer and expression (as assessed by GFP fluorescence). Fluorescence was restricted to malignant tissue. In the experimental group (GCV treated), 8/12 remain alive and well >120 days post glioma implantation. The vTKiGFP is very efficient at transducing human glioma cell lines *in vitro* and leads to significant GCV sensitization. Recombinant retroviral particles can be concentrated to titers which allow *in vivo* intra-tumoral delivery of large viral doses. The therapeutic efficiency of this reagent has been demonstrated in a pre-clinical model of brain cancer

MATERIALS AND METHODS

Cell lines and plasmids.

pCMMP-LZ plasmid (Jeng-Shin Lee and Richard C. Mulligan, unpublished), pJ6Ωbleo plasmid and 293GPG retroviral packaging cell line were generous gifts from Richard C. Mulligan (Children's Hospital, Boston, MA). MSCV-Neo plasmid (36) and BSICZSVPA plasmid (37) were kindly provided by Robert G. Hawley (The Toronto Hospital, Toronto, ON). SKI-1, SKMG-4, SKMG-1, T98G, UW28 & UWR7 human glioma cell lines were generously provided by Lawrence Panasci (Lady Davis Institute for Medical Research, Montreal, QC). C6 & C6/lacZ glioma cells originate from ATCC. pMC1TK plasmid was graciously provided by Gerald Batist (Lady Davis Institute for Medical Research, Montreal, QC). HaL22Y plasmid was kindly provided by Raymond L. Blakley (St. Jude Children's Research Hospital, Memphis, TN).

Retrovector design and synthesis.

A plasmid encoding for a bicistronic, non-splicing murine retrovector which incorporates a multiple cloning site - allowing insertion of cDNA of interest - linked to the Enhanced Green Fluorescence Reporter (AP2) was engineered. The synthesis of AP2 is as follows. The 805 bp EGFP cDNA was excised by Eco47-3 and NotI digest of pEGFP-N1 (Clontech, Palo Alto, CA) and ligated into the MSCV (36) retroviral plasmid to generate MSCV-EGFP. The 555 bp Internal Ribosomal Entry Site (IRES) was excised from the BSICZSVPA

plasmid (37) by SacII-NcoI digest and cloned in to SacII-NcoI cut MSCV-EGFP to generate MSCV-IRES/EGFP. MSCV-IRES/EGFP was digested with SpeI-AscI to generate a 2524 bp fragment encompassing part of the 5' untranslated region of the retrovector, the IRES, EGFP and most of the 3' LTR. This insert was ligated with a 4169 bp fragment from SpeI-AscI cut pCMMP-LZ - an unpublished MFG-based retrovector - to generate AP2 (Fig. 1A). AP2 is designed to co-express an inserted cDNA with the EGFP reporter within a bicistronic framework. The EGFP serves as a reporter of provirus transfer and expression in target cells. The viral vector generated is non-splicing. The pMC1TK plasmid was cut with BglII-BsaWI to generate a 1207 bp fragment containing the HSVTK cDNA (excluding polyadenylation signal) and was ligated into BglII-XmaI-cut AP2 to generate pTKiGFP (Fig. 1B). The retroviral genome produced from pTKiGFP will not incorporate the CMV promoter element. Transduction of target cells with pTKiGFP-derived retroviral particles (vTKiGFP) will lead to the stable incorporation of LTR flanked proviral genome (Fig. 1C). The pMSCV-DHFR (L22Y)/IRES/EGFP vector (pMSCV-DIG) was derived by incorporating the 654 bp BamH1-Xho1 DHFR (L22Y) cDNA from Ha-L22Y into BglII-SalI cut MSCV-IRES/EGFP.

Production of VSVG-pseudotyped retroviral particles and concentration.

Recombinant VSVG-pseudotyped retroparticles were generated either by transient or stable transfection of the 293GPG packaging cell line (34). 293GPG cells are maintained in 293GPG media [DMEM (Gibco-BRL, Gaithesburg, MD), 10% heat-inactivated FBS (Gibco-BRL) supplemented with 0.3 mg/ml G418 (Mediatech, Herndon, VA) and 2 µg/ml puromycin (Sigma, Oakville, ONT), 1 µg/ml tetracycline (Fisher Scientific, Nepean, ONT) and 50 units/ml of Pen-Strep]. For transient production of retroparticles, 293GPG cells were transfected with 5 µg plasmid retrovectors with the use of lipofectamine (Gibco-BRL). Transient transfections were done in tetracycline-free media and viral supernatant collected daily for 1 week, 3 days following transfection. Stable producer cells were generated by co-transfection of 4µg FspI linearized retrovector plasmid and 1:25 ratio of pJ6ΩBleo plasmid. Transfected cells were

subsequently selected in 293GPG media supplemented with 100 µg/ml Zeocin (Invitrogen. San Diego, CA) as described (34). Resulting stable polyclonal producer populations were utilized to generate high titer virus. All viral supernatants were filtered with 0.45 micron syringe mounted filters (Gelman Sciences, Ann Arbour, MI) and stored at –20°C. Concentration of VSVG retroparticles was performed as previously described (34) In brief, previously harvested supernatant was thawed and 10 ml aliquots were centrifuged at 25.000 rpm in a SW41T1 rotor (Beckman Instruments Inc.) at 4°C for 90 minutes. Viral pellets were resuspended overnight in 100 µL serum-free RPMI (Gibco-BRL) at 4°C, pooled and concentrated through a second centrifugation. Concentrated virus was aliquoted and stored at –80°C. Viral preparations were devoid of RCR by EGFP marker rescue assay utilizing supernatant from transduced UWR7 cells.

Transduction of glioma cells. flow cytometry and southern blot analysis.

Human glioma cell lines were plated at $2x10^4$ cells per well in a 24 well dish and allowed to adhere. Media was removed and replaced with 500 µL of thawed, retrovirus conditioned media collected from transiently transfected 293GPG. Polybrene (Sigma) was added to a final concentration of 6 µg/ml. This procedure was repeated daily for three consecutive days Stably transduced cells were subsequently expanded. No clonal selection was performed, and mixed populations of transduced cells were used for all subsequent experiments. Flow cytometric analysis was performed within two weeks following transduction to ascertain retrovector expression and gene transfer efficiency as measured by GFP fluorescence. In brief. adherent transduced cells were trypsinized and resuspended in RPMI at ~10^5 cells per ml. Analysis was performed on a Epics XL/MCL Coulter analyzer. Live cells were gated based on FSC/SSC profile and analyzed for GFP fluorescence. Southern blot analysis was performed on 15 g of overnight NheI digested genomic DNA extracted from stably transduced cells as well as untransduced control cells. Blots were hybridized with a P32 labeled, full-length 700 bp GFP cDNA probe, washed and exposed on photographic film.

Growth suppression assays.

Stably transduced test and control cells were trypsinized and plated at a density of 1000 cells per well in a flat bottomed tissue-culture treated 96 well plate (Costar corporation, Cambridge, MA). Clinical-grade gancyclovir (GCV, Hoffman-Laroche, Mississauga, ONT) was added to achieve a range of concentrations from 0.01 to 5000 µg/ml in a final volume of 100 µL of RPMI/10% FBS. Cells were incubated at 37°C and media was replaced with fresh GCV after three days for a total exposure of 6 days. The percentage of surviving cells was measured using a method based on the metabolism, by living cells, of the mitochondrial substrate 3- (4,5-dimethylthiazol-2-yi)-2,5-diphenyltertrazolium bromide (MTT) into formazan, which is detected by measurement of the optical density at 570nm. Percent Survival is calculated as follows [OD570 test - OD570 empty well]/[OD570 untreated cells - OD570 empty well] x 100. All data points were measured in triplicate in at least three separate experiments.

Titration of retrovector.

Target glioma cells were plated at $2x10^5$ cells per well in a 6 well tissue culture dish. The next day, cells from a test well were trypsinized and enumerated to determine baseline cell count at moment of virus exposure. Virus was serially diluted (range 100 to 0.001 µL) in a final volume of 1 ml of RPMI/10% FBS supplemented with 6 µg/ml polybrene (Sigma) and applied to adherent cells. Flow cytometric analysis was performed 3 days later to determine the percentage of GFP− cells. Viral titer (cfu/ml) was extrapolated from the test point in which non-saturating transduction conditions prevailed (i.e. transduction efficiency <80%). Titer (cfu/ml) was calculated as [(% GFP+ cells) X (cell number at initial viral exposure) . (viral volume in ml applied)].

Animal model of brain cancer. *In vivo* retrovector delivery and gancyclovir treatment.

C6/lacZ glioma cells reproducibly generate lethal intra-cerebral tumors when injected in Sprague-Dawley rats. The constitutive β-galactosidase expression facilitates delineation (by X-gal staining) of tumor cells and extent of the tumor infiltrate in post-mortem brain sections. Adult Sprague Dawley rats

were anesthetized with intraperitoneal injection of ketamine (50 mg/kg) and xylazine (2 mg/kg). C6/lacZ rat glioma cells ($2x10^5$ cells in 5 µl of HBSS) were injected intracranially into the frontal lobe using a Hamilton syringe in a stereotactic apparatus (Kopf) over a period of 15 minutes. The coordinates used were 3.5 mm lateral to the bregma, 1.0 mm posterior to the coronal plane and 4.5 mm in depth of the dural surface. Six days post glioma cell implantation, rats were anesthetized and vTKiGFP (concentrated stock of $2.3x10^{10}$ cfu/ml) was injected of into six different sites (1 mm apart) in the pre-established tumor guided by the previous stereotactic coordinates. A total volume of 9 l was injected in each tumor (6 x 1,5 l increment) and needle was left in place for at least 5 mins per increment (for a total of 30 mins per tumor). Two days after retrovector delivery, rats are treated with GCV 50 mg/kg intraperitoneally twice daily for 5 days followed by 50 mg/kg once daily for another 5 days. After euthanasia, brains were removed and quickly frozen in isopentane chilled with liquid nitrogen. Coronal sections (10 µm) were prepared. GFP activity was observed by epi-fluorescence microscopy and recorded photographically. Subsequently, sections were stained histochemically for β-galactosidase activity as previously described before counter staining with hematoxylin and eosin.

RESULTS

Retrovector design and synthesis.

The AP2 expression vector (Fig. 1A) allows the incorporation of a cDNA sequence in a Multiple cloning site (MCS) upstream of an Internal Ribosomal Entry Site (IRES) and the Enhanced Green Fluorescent Protein (EGFP) cDNA. The transcription initiation from a CMV promoter will lead to the production of a bicistronic mRNA incorporating both the inserted cDNA and the EGFP coding sequence. Translation of both coding sequences will be achieved from a single mRNA molecule, thereby ensuring co-dominant expression of both protein products. Live cells expressing EGFP, which is detectable by fluorescence microscopy or flow cytometry, will co-express the linked gene product. Gene-modified cells can be implanted or transplanted in animal models and their localization and function be traced based on the expression of the EGFP

protein. The AP2 expression vector incorporates a replication-defective retroviral packaging sequence and a retroviral 3' long terminal repeat (LTR). Transfection of an appropriate retroviral packaging cell line can lead to production of recombinant retroviral particles. Retroparticles can be generated either by transient transfection of packaging cell lines or alternatively, stable producer cell lines can be generated by co-transfection with a drug resistance plasmid. We have generated retroparticles by both methods with good success utilizing the 293GPG retroviral packaging cell line.

Retrovector transfer and expression in human glioma cell lines.

The 293GPG packaging cell line was transiently transfected with pTKiGFP (Fig. 1B) and supernatant containing VSVG-typed retroparticles (vTKiGFP) was subsequently collected, filtered and frozen for storage. Human glioma cell lines (SKI-1, SKMG-4, SKMG-1, T98G, UW28 & UWR7) were transduced with three consecutive daily applications of thawed vTKiGFP supernatant. Six days post-transduction, polyclonal cell lines were subjected to flow cytometric analysis to determine the proportion of cells which expressed the GFP reporter protein. All polyclonal cell lines were 100% GFP-positive by FACS analysis, and transduced UWR7 cells serve as a representative example (Fig. 2). We have also found that GFP expression could be easily detected in live cultured cells by direct visualization with a tissue culture microscope fitted with an epifluorescence light source (data not shown). Southern blot analysis confirmed that unrearranged vTKiGFP vector integrated in chromosomal DNA of transduced target cells (Fig. 3). vTKiGFP transduced cells have been passaged in excess of 30 times without loss of GFP expression.

vTKiGFP Expression and Gancyclovir sensitization.

HSV TK expression will lead to the conversion of the prodrug gancyclovir to its cytotoxic metabolite gancyclovir monophosphate. Cells which do not express this enzyme are refractory to gancyclovir toxicity. We compared the gancyclovir sensitivity of vTKiGFP transduced cells with unmodified parental cells as well as cells modified with a control, GFP-containing retrovector (vMSCV-DIG). Cells were plated in 96 well dish and exposed to gancyclovir for a period

of 6 days. Live cell content was assessed colorimetrically by MTT assay and cell survival was expressed as a percentage relative to untreated cells. We have found that all vTKiGFP-expressing cell lines were sensitized to gancyclovir. Comparing the GCV concentration which inhibits cell growth by 50% (IC50), we found that vTKiGFP transduced cells (all 6 cell lines aggregated) were up to 10.000 fold more sensitive to GCV than controls (IC50 tests: 0.004 ug/ml vs IC50 controls: 40 ug/ml, $p<0.001$ by student t test) (Fig. 4). Growth rate for transduced and parental cell lines in the absence of gancyclovir were identical (data not shown).

Concentration of vTKiGFP retroparticles.

The most direct means of transducing a tumor *in vivo* is to inject the therapeutic retrovector intra-tumorally. If the aim is to transduce as many tumor cells as possible, it would be desirable to inject a concentrated vector stock to achieve a high local MOI. We determined if viable vTKiGFP retroparticles could be concentrated by ultracentrifugation as previously described. As a first step we transfected 293GPG cells with pTKiGFP and a zeocin resistance plasmid (pJ6bleo). A stably transfected, Zeocin-resistant polyclonal producer cell population (293AP3) was generated. Flow cytometric analysis for GFP fluorescence revealed that 42% of this mixed population stably expressed the pTKiGFP vector DNA (Fig. 5). Tetracycline withdrawal from the culture media will lead to the production of VSVG-typed vTKiGFP retroparticles. We collected retroparticle-containing media daily from the 293AP3 producer cells from days 3 to 8 following tetracycline withdrawal. Supernatant was cleared of cellular debris with a 0.45u filter and frozen. We have noted that twice daily media collection - as opposed to once daily - doubled the yield of retroparticles from producer cells following tetracycline withdrawal (data not shown). Media were thawed, pooled and subjected to ultracentrifugation as described in Materials and Methods. Supernatant was concentrated 84 fold (20 mls to 0.24 ml) by ultracentrifugation. The concentration step raised titer from 2.9x107 cfu/ml to 220x107 cfu/ml as measured on UWR7 human glioma cells (Fig. 6). 84X concentrates were pooled and subjected to a second ultracentrifugation to achieve a final 1000X (100 ml initial volume to 0.1 ml final volume) concentration. Titer of 1000X retrovector

was 2.3x1010 as determined on rat C6 glioma cells (Fig. 6). Concentrated retrovector aliquots were stored at −80°C until further use. We have observed that unmanipulated (unconcentrated) supernatant from tetracycline-deprived 293GPG producer cells can be toxic to target cells if applied repeatedly. However, no toxicity was observed on target cells if concentrated supernatant was used for transduction purposes, even at the highest tested MOI (>100).

Retrovector expression following intra-tumoral injection of concentrated vTKiGFP retroparticles.

Implantation of C6/lacZ glioma cells will reliably lead to the establishment of intra-cerebral tumors in immunocompetent Sprague-Dawley rats. This cell line will generate large local tumors which are uniformly lethal within 60 days following the initial stereotactic injection of 2x104 cells. Furthermore, C6/lacZ cells constitutively express β-galactosidase which permits the assessment of tumor extent and local invasion in X-gal stained post-mortem brain sections. 18 rats received 2x104 C6/lacZ cells via stereotactic injection in the right brain hemisphere. Six days later, 9 µL of 1000X vTKiGFP retrovector (2×10^{10} cfu/ml) was injected at the tumor site using the same stereotactic coordinates. Of these 18 rats, 6 were randomly chosen and treated with saline. Saline-treated control rats had an average survival of 38 days (range 20 to 52 days). Post-mortem examination of brain revealed macroscopic intra-cerebral tumors, except for 1 rat which died with leptomeningeal tumor spread 8 days after tumor injection (which was excluded from further analysis). Examination of fresh frozen brain sections by epifluorescence microscopy shows that in all animals, a predominant proportion of glioma cells fluoresce green (Fig 7A), including distant micrometastasis. Normal surrounding brain tissue is bereft of green fluorescence. No green fluorescence was observed in untransduced brain tumors (Fig 7C).

Gancyclovir treatment of rats with vTKiGFP-targeted gliomas.

Of 18 rats having received intra-tumoral vTKiGFP retrovector, 12 were subsequently treated with gancyclovir. Two days following retrovector injection, rats received gancyclovir 50 mg/kg intra-peritoneally twice daily for 5 days followed by 50 mg/kg once daily for another 5 days. Significant gancyclovir

toxicity including transient limb paresis and otorhagia, was noted in some rats in the week following GCV treatment. Of 12 gancyclovir treated rats, two died within 10 days following drug treatment presumably from direct GCV toxicity (both animals had brain tumors <1mm in diameter on post-mortem). The other 10 rats fully recovered from GCV toxicity. Two rats developed tumor relapses at the initial injection site and died of progressive disease at day 82. Examination of brain tissue sections on these late relapses. revealed focal GFP expression in the tumors (Fig 7E). Significantly enhanced survival was obtained as eight of 12 GCV-treated test rats (66%) remain long term survivors (>120 days). A supplementary control cohort of 6 rats implanted with C6/lacZ, but without subsequent retrovector administration, was treated with the same GCV regimen. These controls had an average life span of 47 days (range 31 to 63 days) (Fig. 8). With our experimental C6 glioma model , we have not observed a significant difference in average survival between the two control groups (saline controls vs GCV-treated null tumors, p=0.37 (Student t test)) suggesting that GCV treatment, on its own. does not have a measurable impact on survival. as has been suggested by others using 9L glioma implants. These differences may be due different biological properties of these two experimental glioma models.

DISCUSSION

Engineering tumor cells to express the Herpes Simplex Virus Thymidine Kinase cDNA will lead to their destruction if they are subsequently exposed to non-toxic nucleobase analogs such as gancyclovir. This "suicide" effect is accompanied by "bystander" toxicity on adjacent tumor cells not expressing TK, so that a minority of engineered tumor cells - perhaps no more than 10 to 25% - will lead to 100% tumor eradication. Clinical application of this therapeutic strategy requires relatively high efficiency TK gene transfer to pre-established tumors. Furthermore, "collateral" gene transfer to normal adjacent normal tissue would have to be curtailed to prevent GCV toxicity to normal brain tissue.

The affinity of recombinant retroparticles for target tissue is defined by the env protein. Murine amphotropic retroviruses, from which are derived many of the therapeutic retrovectors in glioma targeted gene delivery, will only bind

target cells which express a specific inorganic phosphate transporter. If a target tumor does not express the retrovirus receptor, gene transfer - and therapeutic benefit - is unlikely to occur. Retroparticles which are pseudotyped with the VSVG protein will adopt the wide host range of the vesicular stomatitis virus. The putative VSVG receptor on target cells - which is believed to be membrane phospholipid - is ubiquitously found in all eukaryotic cells. This has led the use of VSVG-pseudotyped retrovectors as gene delivery vehicles in wide assortment of mammalian, non-mammalian and invertebrate cells (47). A major advance in pseudotyping retrovectors with VSVG was achieved when a practical "transient" VSVG retroviral packaging cell line was designed. The subsequent publication of a reliable "stable" high-titer VSVG packaging cell lines - including 293GPG (34) - has allowed the development and characterization of pseudotyped retrovectors for a wide variety of gene transfer applications (52), including tumor cell-targeted gene delivery.

We have examined the utility of a VSVG-pseudotyped suicide retrovector for glioma-targeted gene delivery. To facilitate analysis of vector transfer efficiency and expression in target cells, we engineered a retroviral expression vector which incorporates HSVTK and the EGFP reporter cDNA within a bicistronic transcript (pTKiGFP). We have found that co-dominant expression of the HSVTK cDNA and of the EGFP reporter facilitates a wide assortment of procedures associated with synthesis and characterization of viral vectors. Among these, are the ability to measure endpoint titer from stable retroviral producer cells (Fig. 6) as well as potential use for selecting GFP+ producer cells with a cell sorter device. We have also found that the EGFP reporter can serve as a sensitive marker of retrovector expression in targeted tissue *in vitro* (Fig. 2) as well as *in vivo* (Fig. 7).

We generated a stable retroviral vTKiGFP producer cell line (293AP3) derived from the 293GPG packaging cell line (Fig. 5). Upon tetracycline withdrawal, this retroviral producer cell line will express the VSVG envelope protein and generate pseudotyped retroviral particles. We found that VSVG-pseudotyped retroparticles incorporating vTKiGFP will lead to high efficiency retrovector

transfer to human glioma cell lines *in vitro*. In contrast with standard transfection techniques, or with the use of more "standard" retroviral pseudotypes, we have not required dominant selection of subpopulations of cells to achieve 100% transgene-positive cell populations. Retroparticle conditioned media collected from 293GPG cells transiently transfected with pTKiGFP, was used to generate vTKiGFP transduced glioma cell lines. We noted that transducing glioma cells with concentrated retrovector with a single application at a MOI of ~5 led to more than 90% gene transfer in targeted cells (Fig. 6). Gene expression was durable as assessed by persistent GFP expression (>30 passages) and by functional HSVTK expression, rendering VSVG-associated pseudotransduction unlikely. Having generated vTKiGFP transduced cell lines, we confirmed that the proviral genome integrated unrearranged by southern analysis, demonstrating the stability of the viral vector as designed (Fig. 3) This of some importance especially in light of recent reports documenting rearranged "suicide" retroviral vectors as a cause of gancyclovir resistance in transduced tumors (55). Virtually all glioma cell lines transduced with vTKiGFP acquired substantial and significant sensitivity to gancyclovir *in vitro* (Fig. 4). Our experimental design based on the use of polyclonal transduced cell populations for cytotoxicity assays, supports the hypothesis that vTKiGFP gene transfer, on the average, will express biologically significant levels of TK in a gene-modified cell. Neither the transduction process (with a control retrovector), nor expression of the GFP reporter, on their own, sensitizes cells to gancyclovir (Fig. 4).

Important characteristics of VSVG pseudotyped retroparticles are their ability to sustain concentration by ultracentrifugation and repeated freeze/thaw without loss of activity. These properties have allowed us to collect retroparticle conditioned media on a daily basis following tetracycline withdrawal from the 293AP3 producer cell line. Retroparticle-containing media was frozen and stored until further use. Large volumes of frozen supernatant can be thawed, pooled and subjected to at least two cycles of centrifugation with efficient retrovector recovery. We concentrated 100 mls of media to a final volume of 0.1 ml (1000X concentration on volume basis) This was accompanied by a 800 fold increase in

titer from 2.9 to 2300x107 cfu/ml. We noted that supernatant from tetracycline-deprived 293AP3 producer cells could be toxic to target cells if applied repeatedly. We also observed this phenomena with other 293GPG-derived producer cells (data not shown). Interestingly, we observed that concentrated retroparticles, which had been resuspended in serum-free media did not have this property although they would be delivered at a MOI higher than that achievable with the unconcentrated supernatant. This suggests that supernatant from tetracycline-deprived 293GPG cells contains toxic constituent(s) which are readily discarded upon concentration procedure.

To test the therapeutic usefulness of this reagent, we utilized a rodent model of brain cancer. We established C6/lacZ glioma tumors in immunocompetent Sprague-Dawley rats and subsequently administered concentrated vTKiGFP retrovector intra-tumorally. Intra-tumoral delivery of 9µl (~108 retroparticles) of concentrated vTKiGFP retrovector stock did not improve survival of animals who did not subsequently receive gancyclovir. These control rats (tumor+, retrovector−, but no GCV) had a mean survival of 38 days (range 20-52 days). Post-mortem examination of whole brain tissue sections, revealed that efficient and stable tumor-specific gene transfer had occurred (Fig. 7). Transgene expression persisted in the growing tumor as long as rats survived after retrovector administration (data not shown). Examination of surrounding normal brain tissue failed to reveal GFP fluorescence (Fig. 7) suggesting that retrovector integration and expression occurred in tumor cells only and not in mitotically quiescent neurons, as would be expected from a retroviral vector.

Twelve test rats received GCV following tumor-targeted vTKiGFP delivery. Of these, two died shortly (within two weeks) following the end of GCV treatment. This "acute" death rate attributable to direct GCV toxicity (~16%), is comparable to that observed by other investigators who administered GCV at equal or lesser doses. The mechanism of death is likely related to cytopenia and immunosuppression associated with severe, albeit reversible, bone marrow toxicity. Surviving test rats fully recovered from GCV toxicity approximately two weeks following its completion.

All of the test rats remained alive and well more than 80 days post tumor implantation. Two rats developed symptomatic tumor recurrences and were sacrificed on day 82 post tumor implantation (Fig. 8). Examination of brain tissue sections on these late relapses, revealed large tumors with areas of green fluorescence inter-spaced with GFP-negative tumor cells (Fig 7). This suggests that recurrence was due in part to growth of untransduced tumor cells, or of tumor cells in which the retrovector was silenced following integration. The presence of GFP+ tumor cells suggests that the GCV regimen was not intensive and/or durable enough to eliminate all transduced tumor cells in these rats. Alternatively, a subset of transduced, TK-expressing cells may have acquired resistance to gancyclovir via some other means. Lastly, the "bystander" effect - especially its immune effector arm - may vary in intensity from animal to animal. This may explain the observed pattern of late relapses, suggesting that that there was a early "suicide/bystander" effect which led to increased survival but that some tumor cells - transduced or not - "escaped" from the bystander effect and eventually led to a recurrence. However, the sum of the suicide and bystander effect was clearly sufficient to enhance survival of a majority of animals (66%) who received vTKiGFP and GCV. Our observed long-term survival rate (>120 days) is substantially greater than that observed following intra-tumoral injection of TK retroviral producer cells and compares favorably with that obtained with suicide adenovectors, including those incorporating tumor-specific promoter elements.

In the experimental group, 2/12 animals died from GCV toxicity and 2/12 succumbed to late tumor recurrences. These data suggest that GCV dose reduction would be desirable to lessen toxicity, however the duration of treatment may need to be extended to allow elimination of all gene modified cells The relatively late recurrences (day 82 post implant), lead us to speculate that the "immune" bystander effect may have been mitigated in these two animals. It may be possible to increase the immune response by co-administering immunomodulatory genes (IL2, GMCSF) with TK such as has been described by others. Furthermore, it may be useful to re-administer the suicide retrovector to those

animals who have residual disease following a cycle of therapy, and to repeat this until maximal response has been achieved. However, it is unknown if a specific - and neutralizing - immune response against VSVG-typed retroparticles will be elicited.

This constitutes the first report of *in vivo* delivery of a cell-free retrovector concentrate with tumor-specific, high efficiency gene transfer and expression, with evident biologically significant anti-tumor activity. We propose that concentrated vTKiGFP retrovector may be of therapeutic value for humans with brain cancer. The high-titer of the concentrated reagent would allow intra-tumor delivery of a useful retrovector dose without the risks of injecting relatively large volumes in a confined space (such as brain). vTKiGFP targeting of a tumor mass *in vivo* should subsequently lead to its regression, and the bystander effect may have a significant impact on the biology of local and distant micrometastatic glioma deposits within the neuropil. This and related therapeutic reagents may also be useful in the treatment of other locally advanced and metastatic malignancies.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A retroviral particle for delivering a gene to a tumor tissue cell, said retroviral particle being pseudotyped with a vesicular stomatitis virus G (VSV G) protein.

2. A tumor-specific retroviral expression vector comprising a suitable promoter, a retroviral untranslated sequence including a packaging sequence and a primer building site, a cloning site operably linked to an internal ribosomal entry site (IRES), said IRES being operably linked to a first nucleotide sequence encoding a suitable marker, a retroviral 3' long terminal repeat (LTR) sequence, for expressing a second nucleotide sequence inserted in said cloning site.

3. A retroviral expression vector according to claim 2, wherein said second nucleotide sequence comprises a therapeutic gene.

4. A retroviral expression vector according to claim 3, wherein said therapeutic gene comprises a suicide gene

5 A retroviral expression vector according to claim 4, wherein said nucleotide sequence encodes a Herpes simplex virus thimidine kinase.

6. A retroviral expression vector according to claim 5, wherein said marker comprises a green fluorescent protein (GFP).

7. A plasmid encoding a bicistronic, non-splicing murine retrovector comprising a multiple cloning site (MCS) operably linked to an enhanced green fluorescent (EGFP) reporter (AP2) for transferring a provirus to a target cell and expressing said provirus into said target cell, for co-expressing a nucleotide sequence inserted into said plasmid with said EGFP reporter within a bicistronic framework.

8. A replication-defective retroviral expression vector comprising a suitable promoter, a retroviral untranslated sequence including a packaging sequence and a primer building site, a multiple cloning site (MCS) operably linked

to an internal ribosomal entry site (IRES), said IRES being operably linked to a first nucleotide sequence encoding a suitable marker, a retroviral 3' long terminal repeat (LTR) sequence, for expressing a second DNA sequence inserted in said MCS.

9. An expression vector according to claim 8, wherein said marker comprises an enhanced green fluorescent protein (EGFP).

10. An expression vector according to claim 9, wherein said promoter comprises a CMV promoter.

11. A method for treating a tumor, the method comprising administering to a mammal suspected of having a tumor a retroviral expression vector comprising a first nucleotide sequence, said first nucleotide sequence being therapeutic, and a second nucleotide sequence encoding a marker, said first and second nucleotide sequences being co-dorminantly expressed, and administering to said mammal a nucleobase analog

12. A method for detecting *in vivo* a genetically modified cell with an expression vector according to claim 7 to a tumor tissue cell of a mammal, the method comprising administering a retroviral expression vector comprising a first nucleotide sequence encoding a retrovirus and a second nucleotide sequence encoding a marker, said first and second nucleotide sequences being co-dominantly expressed, and detecting the expression of said second nucleotide sequence by using one of fluorescence microscopy and flow cytometry techniques.

13. A method for producing a retroviral particle according to claim 1, the method comprising stably transfecting a suitable cell line with the expression vector of claim 7.

14 A method for producing retroviral particles, the method comprising transfecting a suitable cell line with the expression vector of claim 8 and transfecting said cell line with a drug resistance plasmid.

15. The cell line obtained by the method according to claim 12.

ABSTRACT OF THE INVENTION

The invention relates to retroviral expression vectors and more particularly to pseudotyped retroviral vectors for gene therapy of cancer. Direct *in vivo* tumor-targeting with "suicide" viral vectors is limited by inefficient gene transfer and indiscriminate transfer of a conditionally toxic gene to surrounding non-malignant tissue. Retrovectors pseudotyped with a Vesicular Stomatitis Virus G protein (VSVG) may serve as a remedy to this conundrum. These retroviral particles differ from standard murine retroviruses by their very broad tropism and the capacity to be concentrated by ultracentrifugation without loss of activity. A VSVG-typed retrovector can be utilized for efficient and tumor specific Herpes Simplex Virus Thymidine Kinase (TK) gene delivery *in vivo*. A bicistronic retroviral vector which expresses TK and Green Fluorescence Protein (pTKiGFP) was constructed.

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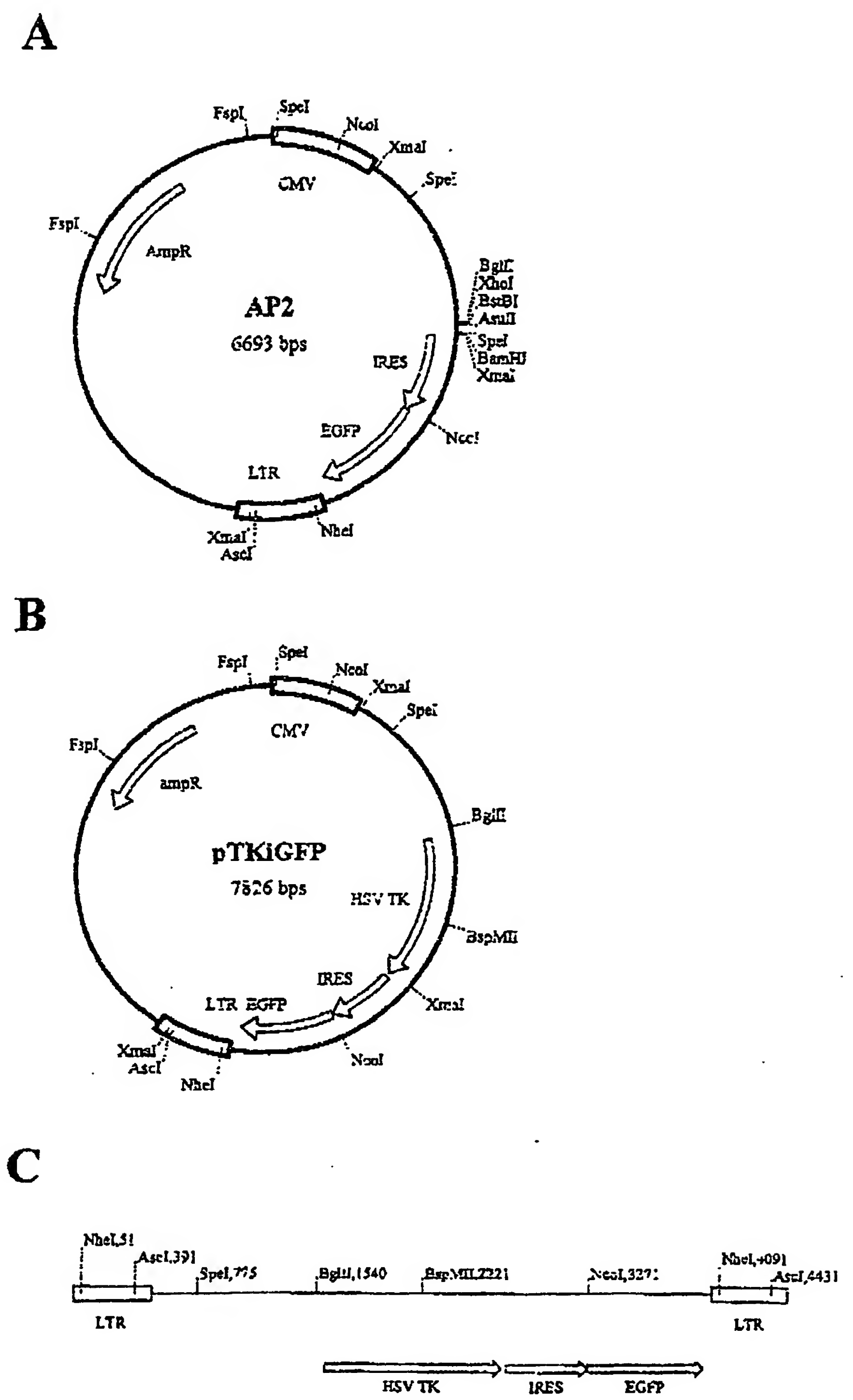
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XmaI
IRES
EGFP
NcoI
LTR
XmaI
AscI
NheI
B
FspI
SpeI
NcoI
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SpeI
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Fig. 1

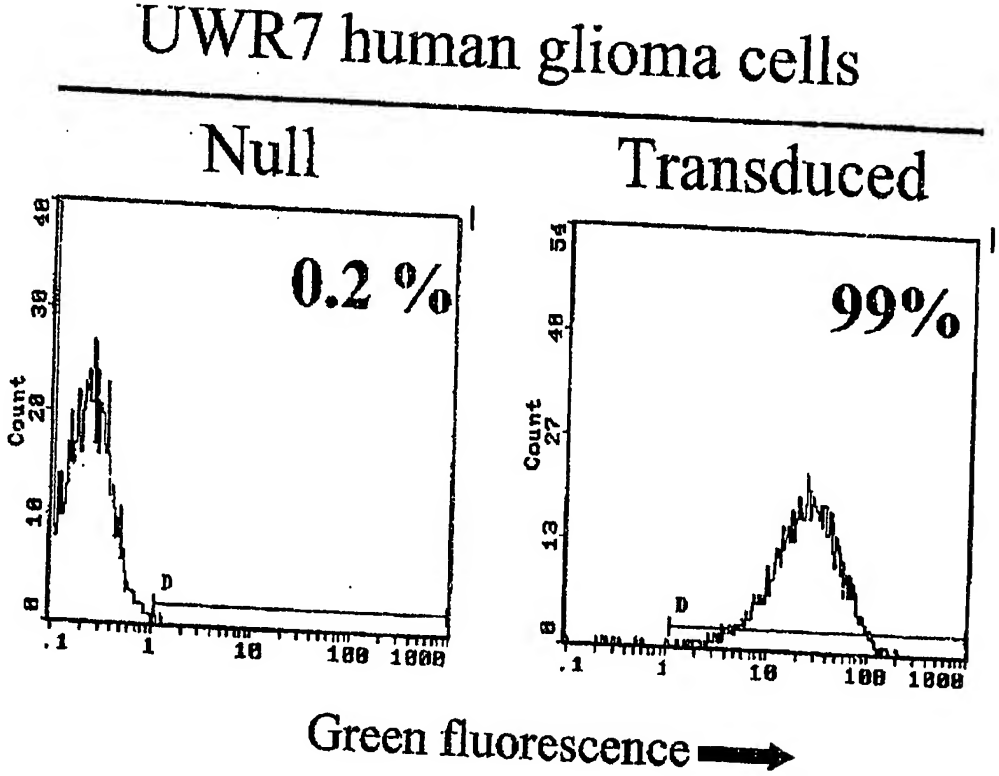


Fig. 2

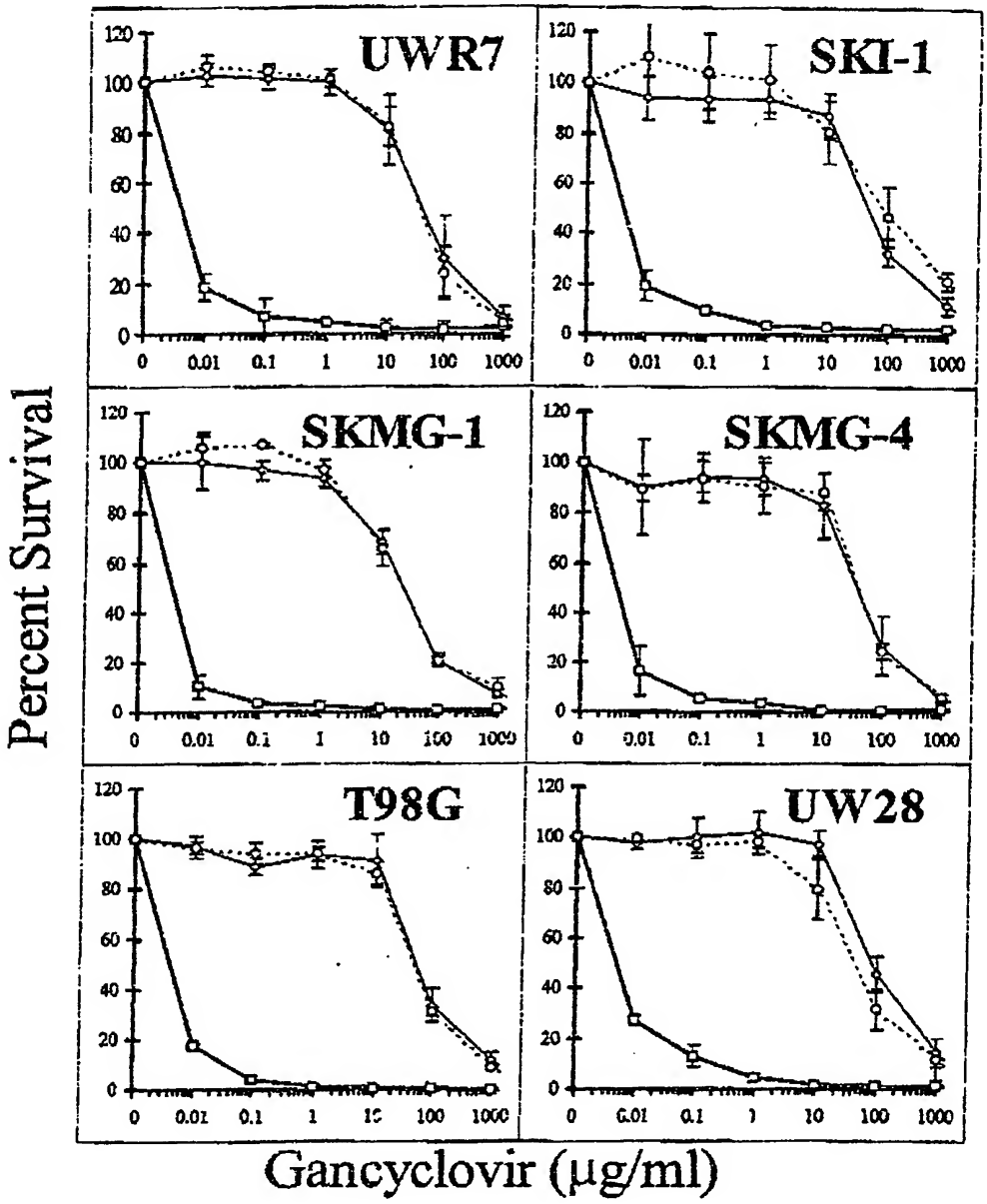


Fig. 4

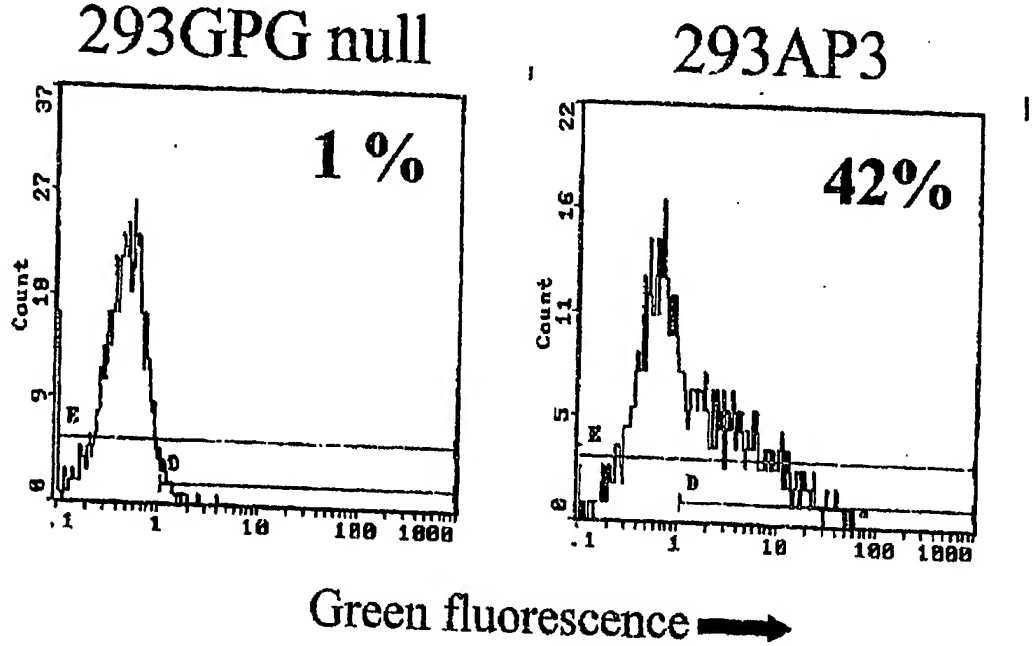
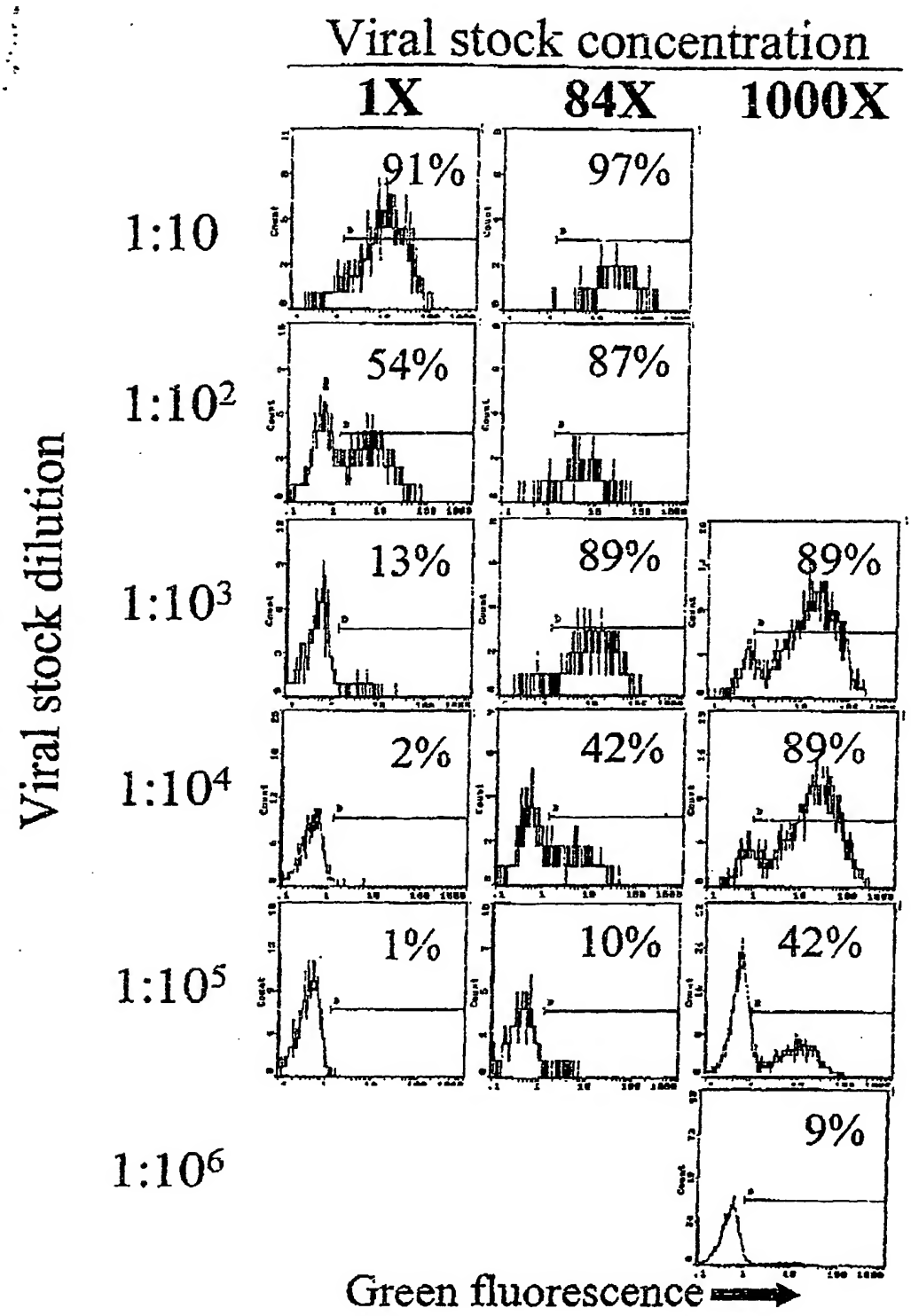


Fig. 5


Viral stock concentration
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84X
1000X
Viral stock dilution
1:10
1:10²
1:10³
1:10⁴
1:10⁵
1:10⁶
91%
97%
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89%
89%
2%
42%
89%
1%
10%
42%
9%
Green fluorescence

Fig. 6

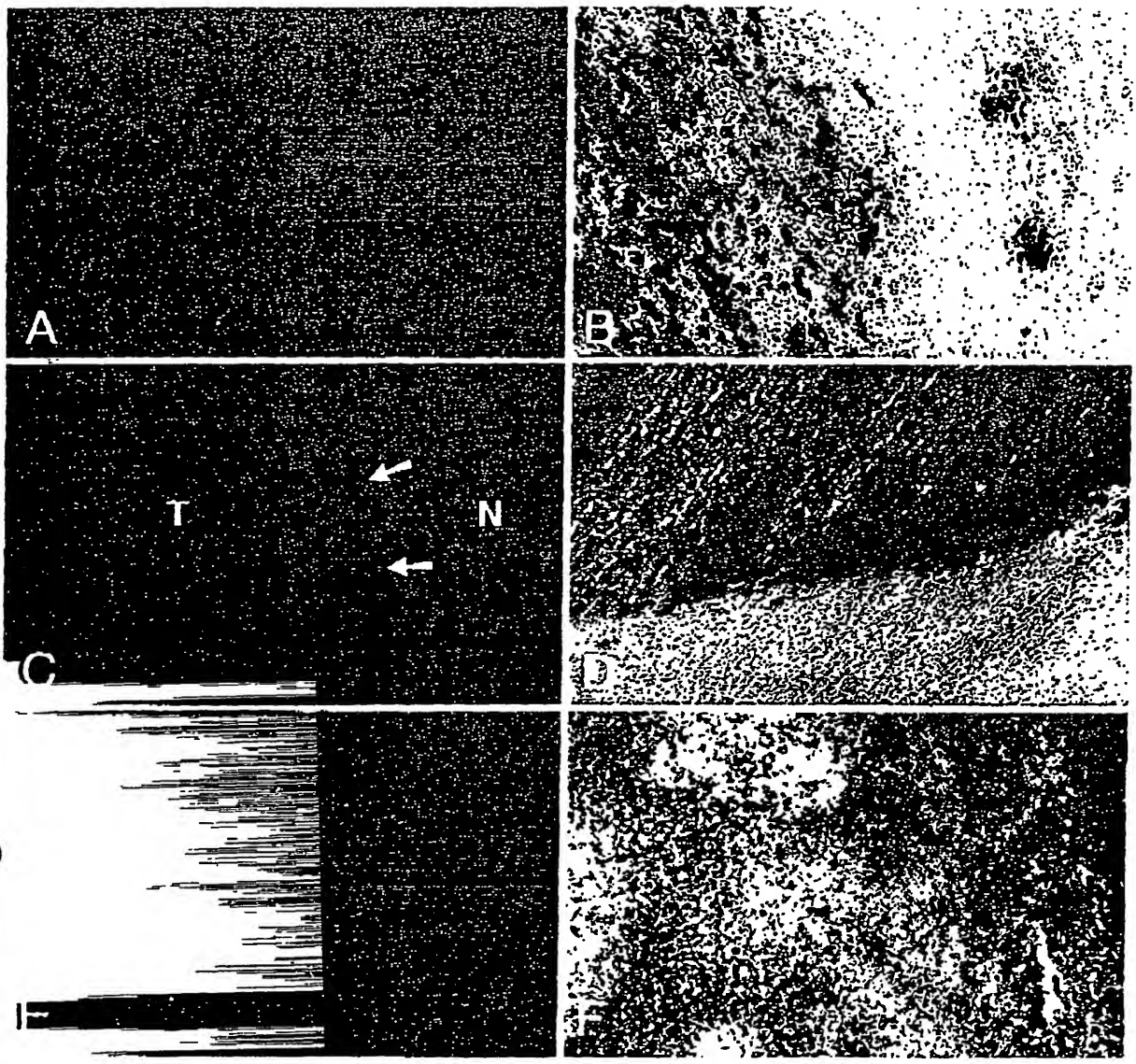

Fig. 7

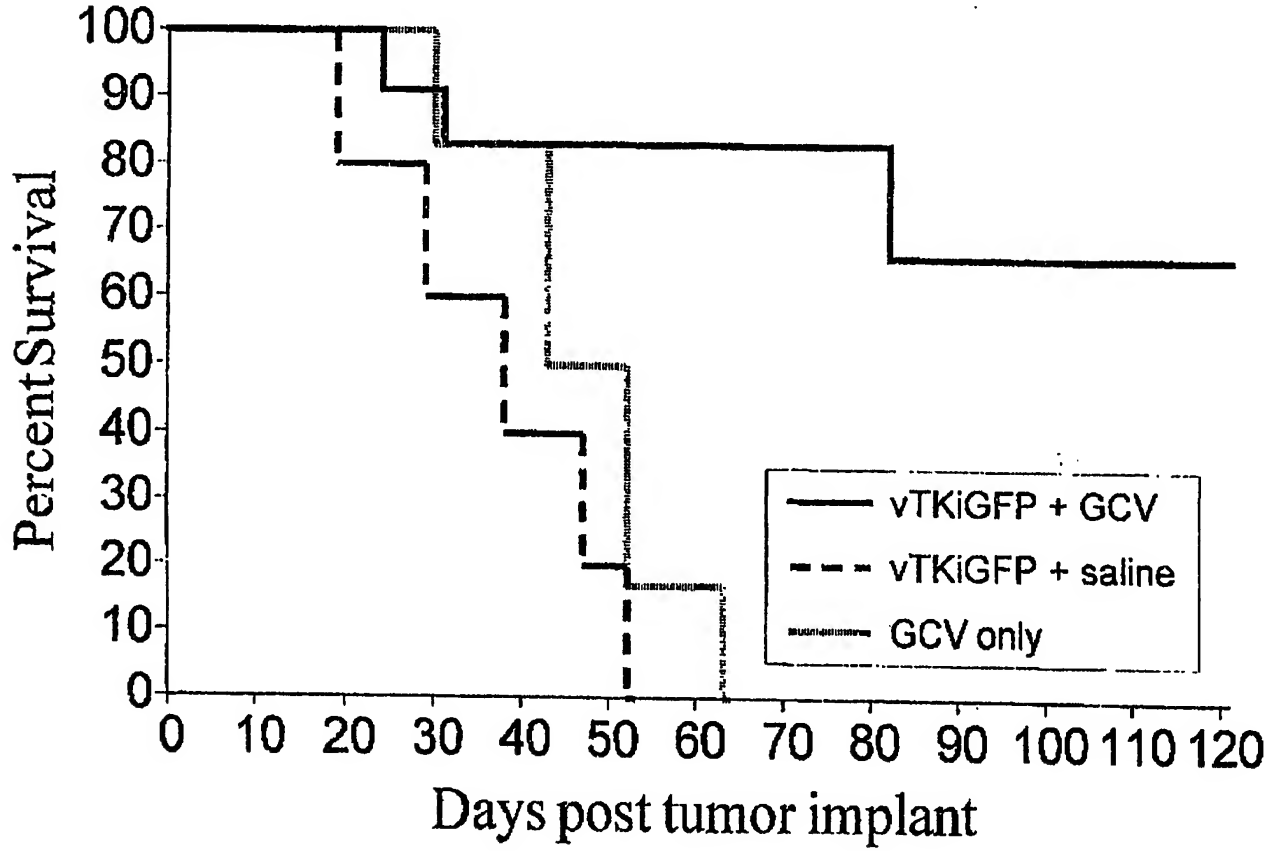

Fig. 8